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Determination of the Phosphorylation, Uncovering of Mannose 6-Phosphate Groups and Targeting of Lysosomal Enzymes

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Summary: There are at least three stages in the targeting of soluble lysosomal enzymes: transfer of N-acetylglucosaminyl 1-phosphate to high-mannose oligosaccharide side chains, removal of N-acetylglucosamine and recognition of the "uncovered" mannose 6-phosphate residues. Defects in the transfer reaction cause mucolipidoses II and III. Those in the subsequent stages of the targeting may result in similar clinical disorders. To differentiate between possible defects of the targeting in cultured cells we have developed a procedure for a combined detection of the phosphorylation, uncovering of the transferred phosphate residues and the targeting of lysosomal enzymes. For this purpose cultured cells are metabolically labelled with [^{32}P]phosphate and a lysosomal enzyme, such as cathepsin D, is isolated from the labelled cells and the medium by immunoprecipitation. The immunoprecipitates are dissolved with sodium dodecylsulphate and incubated in the presence and absence of calf intestine alkaline phosphatase. We show that the treatment of the denatured protein results in hydrolysis of phosphomonoester groups and that the phosphodiester and the peptide bonds remain intact. The initial and the residual radioactivity associated with the lysosomal enzyme which represent the total phosphate and the phosphodiester groups, respectively, are determined by gel-electrophoresis, fluorography and densitometry. This procedure extends one of the previously established methods for the diagnosis of mucolipidoses II and III.

Introduction

The targeting of soluble lysosomal enzymes depends on the acquisition of mannose 6-phosphate groups (reviewed in l. c. (1–3)). These groups are synthesized on high-mannose oligosaccharide side chains of soluble lysosomal enzymes and activator proteins in two steps. In the first step, N-acetylglucosaminyl 1-phosphotransferase[1]) yields a phosphodiester and in the second step the phosphodiester is hydrolysed by the "uncovering" enzyme, N-acetylglucosamine 1-phosphodiester α-N-acetylglucosaminidase[1]). Partial or complete deficiencies in the activity of N-acetylglucosaminyl 1-phosphotransferase cause mucolipidoses III and II, respectively (4, 5). A deficiency in the uncovering enzyme is likely to cause a phenotype similar to mucolipidosis III (6).

In a combination with immunoprecipitation, gel-electrophoresis and fluorography, metabolic labelling has been used successfully for screening of the phosphotransferase activity in cultured mucolipidosis II or III cells (7). In this assay both the labelling and the distribution of a lysosomal enzyme between cells and medium are determined so that defects in either the phosphorylation or targeting can be recognized. We argued that the labelling followed by immunoprecipitation, gel-electrophoresis and fluorography should

[1]) Enzymes
N-Acetylglucosamine 1-phosphodiester N-acetylglucosaminidase (EC 3.1.4.45)
Alkaline phosphatase (EC 3.1.3.1)
Cathepsin D (EC 3.4.23.5)
N-Acetylglucosaminyl phosphotransferase (EC 2.7.8.17)

be useful for simultaneously and differentially determining the phosphodiester and phosphomonoester groups, provided that the latter could be efficiently and specifically removed from the immunoprecipitated enzymes by a phosphatase. In addition to phosphorylation, such a determination would also reveal defects in the uncovering. We elaborated the experimental conditions for this determination using calf intestine alkaline phosphatase, which is commercially available, and U937 cells, which are a relatively rich source of cathepsin D. From this enzyme we also isolated phosphorylated oligosaccharides in an amount sufficient for the separation and quantitation of their phosphomono- and diester forms by ion exchange chromatography (8) and scintillation counting. The ratio of the radioactivity in these oligosaccharides confirmed the value which was determined by the new, much simpler and more sensitive procedure.

Materials and Methods

Cell culture

Human U937 (1,25-dihydroxiferolcalcitriol ihydrox(1,25-dihydroxferolpromonocytes (9) were cultured in RPMI 1640 medium and treated with 10^{-7} mol/l (1,25-dihydroxycholecalciferol) as previously described (10). The treatment resulted in an increased expression of cathepsin D.

Metabolic labelling

In studies on glycopeptide labelling, cells grown to a density of $5-10 \times 10^8$/l were collected by centrifugation, washed and subjected to metabolic labelling as described previously (11), except that RPMI 1640 medium was used. The labelling medium was free of either methionine or phosphate and contained 1.3 GBq/l [^{35}S]methionine or 3.7–7.4 GBq/l [^{32}P]phosphate. The phosphate was carrier-free and the methionine had a specific activity of 50 TBq/mmol. The radioactive chemicals were purchased from Amersham-Buchler, Braunschweig, Germany. In pulse-chase experiments with [^{35}S]methionine the labelling was performed for 24 h and terminated by adding a concentrated solution of methionine to a final concentration of 10 mg/l.

Immunoprecipitation

The cells were collected by centrifugation and taken up in 1 ml of a detergent mixture containing 10 ml/l Triton X-100, 5 g/l Na deoxycholate, 1 mmol/l $MgCl_2$, 5 mmol/l iodoacetamide, 1 mmol/l phenylmethanesulphonyl fluoride, 20 mg/l DNase I, 0.14 mol/l NaCl, 10 mmol/l Na phosphate, pH 7.4. After centrifugation the medium was mixed with 0.2 ml of five-fold concentrated stock solution of the detergent mixture. The samples were subjected to freeze-thawing and centrifugation at 40 000 *g* for 1 h. The supernatants were mixed with affinity purified rabbit anti-human cathepsin D (20 µg per aliquot corresponding to 5×10^5 cells) and goat anti-rabbit immunoglobulin conjugated to Eupergit C1Z (0.5 µg second antibody per µg first antibody). Eupergit C1Z was purchased from Roehm Pharma, Weiterstadt, Germany. The samples were rotated end-over at 4 °C overnight. The immunoprecipitates were collected by centrifugation and washing as described elsewhere (12).

Treatment of immunoprecipitates with alkaline phosphatase

The washed immunoprecipitates were solubilized by heating at 95 °C for 5 min with 50 µl 6 g/l SDS, 60 mmol/l Tris-HCl, pH 8.8. The suspension was diluted with 105 µl H_2O and centrifuged. The supernatant was divided into 50 µl aliquots, which were mixed with 2.5 µl of various dilutions of calf intestine alkaline phosphatase in the same buffer as supplied from Boehringer-Mannheim. Under standard conditions the incubations were performed with 0, 6.2 and 12.5 units enzyme (catalogue No. 567744) for 24 h at 37 °C. The incubations were terminated by adding 25 µl 28 g/l SDS, 28 mmol/l dithiothreitol, 80 ml/l glycerol, 0.35 mol/l Tris-HCl, pH 6.8 and heating. The completeness of the digestion was determined by comparing the results of the two incubations with the increasing amounts of the enzyme; the results were the same when a purer enzyme preparation (catalogue No. 713 023) was used.

A rather high amount of calf intestine alkaline phosphatase was needed to achieve complete cleavage of mannose 6-phosphate residues in lysosomal enzymes. Under our incubation conditions, mannose 6-phosphate (10 mmol/l) exerted a weak probably competitive inhibition (28%) of the hydrolysis of 5.5 mmol/l 4-nitrophenyl phosphate. In choosing the buffer we took into consideration its compatibility with the subsequent analysis in polyacrylamide-gel electrophoresis. SDS, which was used to solubilize the immunoprecipitate, stimulated hydrolysis of 4-nitrophenyl phosphate. In the concentration range 1–10 g/l SDS the activity was increased 1.7 to 2-fold.

Separation and quantification of radioactive glycopeptides

After the above treatments, the immunoprecipitates were subjected to polyacrylamide-gel electrophoresis in the presence of SDS with 131 g/l acrylamide in the separating gel as described previously (11). The radioactivity was visualized by fluorography (13) and quantified by densitometry using an Ultroscan densitometer from LKB-Pharmacia, Freiburg, Germany. Tungsten screens "Lightning plus" from Du Pont de Nemours, Bad Nauheim, Germany, were used for detection of ^{32}P. The radioactive standards were prepared by incubating [^{14}C]KCNO with cytochrome c (M_r = 12 300), carbonic anhydrase (M_r = 30 000), ovalbumin (M_r = 46 000), bovine serum albumin (M_r = 69 000) and phosphorylase b (M_r = 97 400) and dialysis.

Isolation and characterization of oligosaccharides

1×10^6 calcitriol-treated U937 cells were labelled for 24 h in 1.1 ml medium containing 9.3 MBq [^{32}P]phosphate and 10 mmol/l NH_4Cl. Cathepsin D was precipitated from cells and medium. An aliquot of 20% of the immunoprecipitate was used for the standard treatments without and with alkaline phosphatase and 80% was subjected to polyacrylamide gel electrophoresis for the isolation of cathepsin D precursor. Following fluorography the gel area (5 × 9 mm) containing the precursor was cut out, soaked for 2 h in 0.1 ml 60 mmol/l Na acetate, 5 mmol/l Na phosphate, pH 5.3, crushed with a pestle and incubated with 1 mU endo-β-N-acetylglucosaminidase H (Boehringer-Mannheim) for 7 h at 37 °C. Then 0.1 ml 60 mmol/l Na acetate and 1 mU enzyme were added and the incubation was continued for 24 h. The hydrolysed material was collected in 0.4 ml acetate buffer, concentrated by lyophilization and desalted in a spun Sephadex G-10 (LKB-Pharmacia) column in 20 mmol/l NaCl. The radioactive material was aliquoted and one half was hydrolysed at pH 3 (14). Both samples were dried, dissolved in 40 µl 50 mmol/l Tris-HCl, pH 9.0, and halved to be further incubated without or with 4 µg alkaline phosphatase for 20 h at 37 °C. The samples were diluted with 1.4 ml 2 mmol/l ammonium acetate, pH 5.3 and applied to 0.5 × 4 cm QAE-Sephadex A-25 columns that were equilibrated with 2 mmol/l

ammonium acetate (8). The elution was performed in 0.5 ml fractions by increasing the concentration of the buffer by 7 mmol/l at each step. The radioactivity was determined in a liquid scintillation spectrophotometer.

Results

Labelling of cathepsin D with [^{32}P]phosphate and selective cleavage of phosphomonoester residues

We developed a new procedure differentiating between "covered" and "uncovered" phosphate residues in a lysosomal enzyme according to the scheme shown in figure 1. Several experiments controlling the reliability of this method are described below.

When cathepsin D was isolated from calcitriol-treated U937 cells after metabolic labelling with [^{32}P]phosphate, the radioactivity was found in the precursor, intermediate, large mature and small mature polypeptides (fig. 2, lane 1). In the medium the radioactivity was found in the precursor polypeptide (fig. 2, lane 7). A small amount of the radioactive large mature polypeptide was also present and probably represented a release from differentiated or from dead cells. The radioactivity associated with the different polypeptides of cathepsin D was present in their carbohydrate moieties, as indicated by removal of all the label with glycopeptidase F (not shown).

Depending on the polypeptide, a characteristic proportion of phosphate residues were resistant to phosphatase even in the presence of an excess of the hydrolase. With isolated oligosaccharides it was found that this proportion represented phosphodiester groups (see below). The proportion was the same when alkaline phosphatase preparations dedicated for either enzyme-linked immunoassays or molecular biology were used (fig. 2). The phosphodiester groups were more abundant in the precursor than in the mature forms of cathepsin D polypeptides.

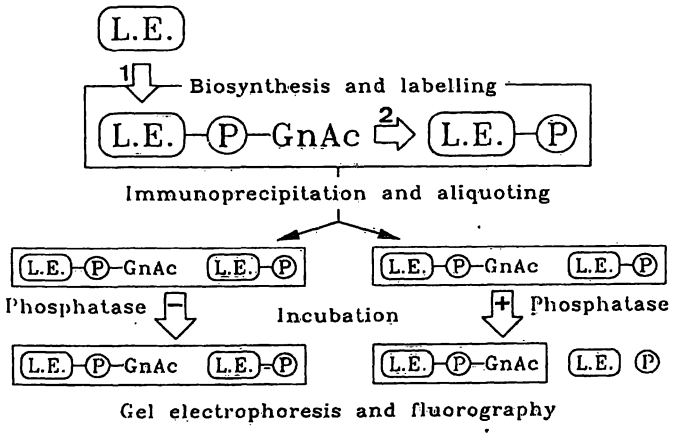

Fig. 1. Scheme depicting the principle of the labelling of, and differentiation between the total and covered phosphate residues in carbohydrate side chains in a lysosomal enzyme. The reactions 1 and 2 denote the two steps in the biosynthesis of mannose 6-phosphate residues catalysed by N-acetylglucosaminyl phosphotransferase and N-acetylglucosamine 1-phosphodiester N-acetylglucosaminidase.
L. E. = lysosomal enzyme; P = radioactive phosphate residue; GnAc = N-acetylglucosamine. The quantitation of the labelled forms of the enzyme is indicated by the rectangular areas.

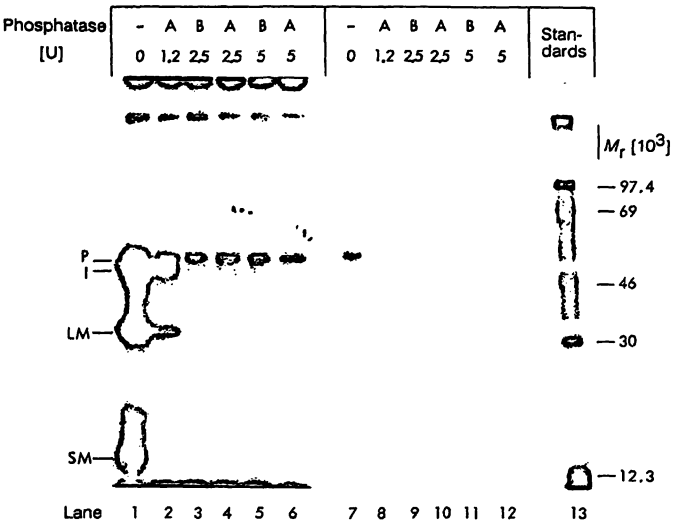

Fig. 2. Digestion of ^{32}P-labelled cathepsin D from calcitriol-treated U937 cells and medium with various amounts of alkaline phosphatase. The cells were labelled with [^{32}P]phosphate for 24 h. Aliquots of the immunoprecipitates of cathepsin D were incubated for 30 h at 37 °C with various amounts of alkaline phosphatase as indicated. A and B refer to calf intestine alkaline phosphatase catalogue Nos. 567744 and 713023, Boehringer-Mannheim, respectively. Radioactivity in the samples was analysed by SDS polyacrylamide gel electrophoresis and fluorography. The positions of the precursor (P), intermediate (I), large mature (LM) and small mature (SM) polypeptides of cathepsin D are indicated.

The removal of phosphate residues from cathepsin D glycopeptides was not due to a proteolytic digestion of the latter. Indeed, treatment of [^{35}S]methionine-labelled glycopeptides did not significantly change the intensity or apparent size of the precursor, intermediate or large mature glycopeptides of cathepsin D (fig. 3). This was not a trivial finding because treatment of the denatured labelled cathepsin D with an alkaline phosphatase from *Escherichia coli* resulted in degradation of the polypeptides (not shown). The small mature glycopeptide was well detected only in immunoprecipitates from calcitriol-treated cells, and its mobility was partially retarded following the treatment (fig. 3, lanes 7–9). It had been noticed earlier that the mobility of the small glycopeptide depended on the composition of its carbohydrate side chain (15).

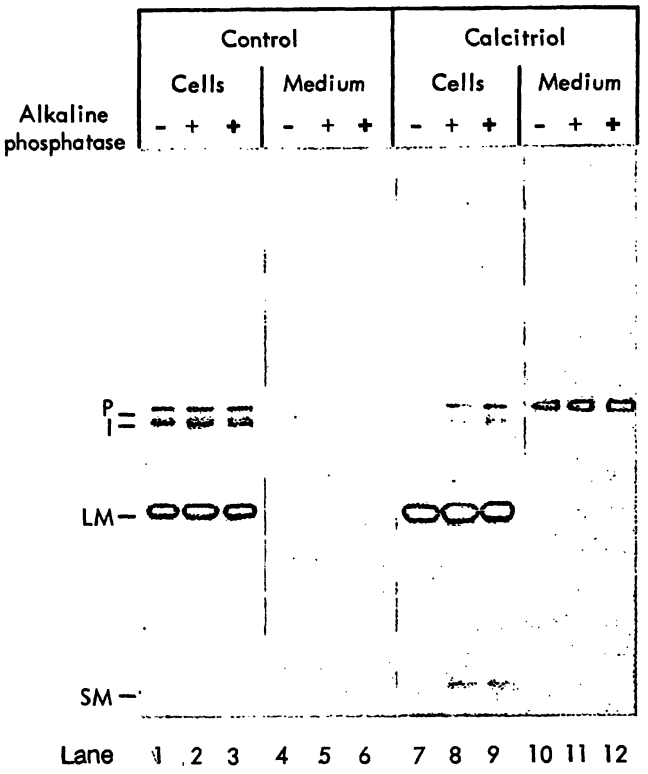

Fig. 3. Effect of alkaline phosphatase on the electrophoretic mobility of [^{35}S]methionine-labelled cathepsin D polypeptides. Control and calcitriol-treated U937 cells were subjected to a 25 h-pulse and 16 h-chase metabolic labelling with [^{35}S]methionine. Cathepsin D was immunoprecipitated from cell and medium extracts and aliquots of the immunoprecipitates were incubated without and with alkaline phosphatase using the standard conditions. The labelled polypeptides were separated by SDS polyacrylamide gel electrophoresis and visualized by fluorography (see legend of fig. 2 for the symbols).

Calf intestine alkaline phosphatase is less active with mannose 6-phosphate than with 4-nitrophenyl phosphate. However, the phosphatase is activated in the presence of SDS, and it is possible to remove the phosphomonoester groups from denatured lysosomal enzymes if a relatively high amount of the phosphatase is used. According to our experience the method is applicable to α- and β-chains of β-hexosaminidase and to α-glucosidase after immunoprecipitation from metabolically labelled fibroblasts (not shown). It can also be used with arylsulphatase A (Dr. *V. Gieselmann*, personal communication) and probably many other lysosomal enzymes.

Proportion of the phosphomonoester and phosphodiester groups in isolated oligosaccharides

From the data presented so far we concluded that a prolonged treatment with the commercial alkaline phosphatase was adequate to ensure a selective, complete hydrolysis of phosphomonoester groups in [^{32}P]phosphate-labelled cathepsin D, with the conservation of phosphodiester groups. To further validate this conclusion, we isolated carbohydrate side chains from the labelled cathepsin D and fractionated the oligosaccharides in a QAE-Sephadex A-25 column. The radioactivity separated into three major peaks that were eluted at 60, 90 and 210 mmol/l ammonium acetate (fig. 4). By analogy to the separation reported by *Lazzarino & Gabel* (8) the material present in peak I was supposed to contain a phosphodiester, peak II a phosphomonoester and peak III two phosphomonoester groups. This tentative assignment was cofirmed by the following observations:

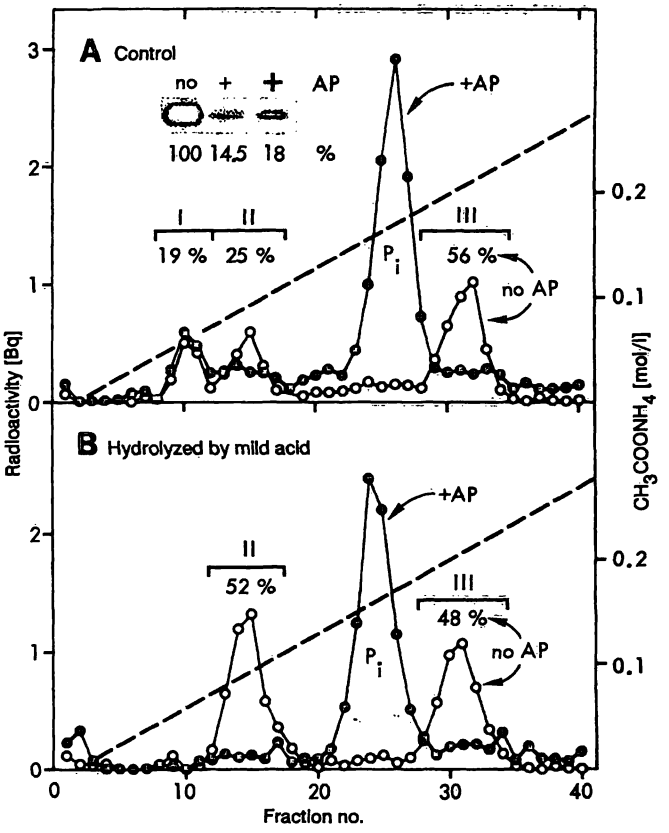

Fig. 4. Characterization of ^{32}P-labelled oligosaccharides from cathepsin D. Calcitriol-treated U937 cells were metabolically labelled with [^{32}P]phosphate in the presence of 10 mmol/l NH_4Cl and the secreted cathepsin D was immunoprecipitated. Oligosaccharides were isolated from 80% of the material using endoglucosaminidase H, and aliquots were treated with either mild acid, alkaline phosphatase (+AP) or both as described in Materials and Methods. The oligosaccharides were separated on QAE-Sephadex A-25 and the radioactivity in the fractions was determined.
A: Separation of oligosaccharides without treatment (○) or after incubation with alkaline phosphatase (●). In the untreated sample the radioactivity was separated into three main peaks. The sum of the radioactivity in fractions marked by the bars was set at 100%. The remainder (20%) of the immunoprecipitate was analysed by the standard procedure for sensitivity to alkaline phosphatase; the fluorogram of the radioactivity in samples that were incubated without or with 6.2 units (+) and 12.5 units enzyme (+) is shown in the inset.
B: The oligosaccharides were treated with mild acid: one half was fractionated directly (○) and the other after incubation with alkaline phosphatase (●).

i) the material present in peak I but not in peaks II and III was resistant to alkaline phosphatase;

ii) material in peak I but not in peaks II and III was sensitive to mild acid hydrolysis;

iii) treatment of the oligosaccharides with alkaline phosphatase converted a large proportion of the radioactivity associated with peaks II and III to a product with the chromatographic mobility of inorganic phosphate, and it was eluted at 170 mmol/l ammonium acetate, and

iv) a complete conversion of the radioactivity to free phosphate was observed only if the oligosaccharides were subjected to mild acid hydrolysis prior to the treatment with alkaline phosphatase.

In control samples the sum of the radioactivity present in peaks I and II was less than in peak III, while following the mild acid treatment the proportions were reversed. It is likely that some of the radioactivity was associated with phosphorylated hybrid oligosaccharides. These were expected to fractionate between peaks II and III or within peak III. In mild acid hydrolysis these oligosaccharides should be converted to phosphomonoester-containing oligosaccharides and be recovered in peak II.

Of the radioactivity present in cathepsin D that was immunoprecipitated from the NH_4Cl-elicited secretion, 14–18% resisted the treatment with alkaline phosphatase (see inset in fig. 4a). Of the radioactivity recovered in the three major oligosaccharide peaks from the same cathepsin D preparation, 19% was present in peak I, i.e. in the phosphatase-resistant and mild acid-sensitive material. Therefore, the treatment of solubilized immunoprecipitates with calf intestine alkaline phosphatase is adequate for discriminating the relative contents of phosphomono- and phosphodiester groups in [^{32}P]labelled cathepsin D.

Discussion

By metabolic labelling of cultured cells with radioactive phosphate, immunoisolation of a labelled lysosomal enzyme from cells and medium and determination of the uncovering ratio of the phosphate groups in this enzyme, it is possible to determine three parameters which are important for its targeting to lysosomes. These parameters are

i) the efficiency of the phosphorylation,

ii) the efficiency of the retention of the phosphorylated enzyme and

iii) the efficiency of the uncovering of its phosphate residues.

In the present work we established the conditions for the determination of the third parameter using commercially available alkaline phosphatase. Under these conditions only the phosphomonoester residues are hydrolysed; the phosphodiester residues and the protein backbone remain intact.

Previously the uncovering of phosphate residues has been determined by ion exchange chromatography of isolated oligosaccharides (8). Unlike the present method, this separation distinguished oligosaccharides containing a single phosphate residue from those containing two. However, this separation is arduous and the sensitivity of the detection of the fractionated oligosaccharides by scintillation counting is an order of magnitude lower than that of the labelled polypeptides by screen-enhanced fluorography, which is approx. 1 Bq ^{32}P.

With the new method small variations in the uncovering of phosphate residues that are likely to occur among different cell strains can be monitored. Using this method we have observed that in fibroblasts the uncovering is diminished and the rate of secretion of cathepsin D with covered phosphate residues is enhanced if the cells are labelled in the presence of NH_4Cl (16). For the determination of the uncovering, any well detectable phosphorylated lysosomal enzyme can probably be used.

The efficiency of the uncovering and targeting of lysosomal enzymes may play an important role in the aetiology of mucolipidoses II and III. *Alexander* et al. (6) reported a clinically normal individual with elevated lysosomal enzyme activities in plasma. Fibroblasts from this individual contained approx. half the normal N-acetylglucosamine 1-phosphodiester N-acetylglucosamidase. The uncovering ratio in lysosomal enzymes from cells with a diminished activity of this enzyme has not yet been examined. The activity of the uncovering enzyme has been determined in very few laboratories. The natural substrates of this enzyme are difficult to prepare (17, 18) and it is not clear if the alternative substrate UDP-N-acetylglucosamine (18) can universally be used. The formation of the "recognition marker" by the two modifying enzymes can be assayed indirectly with the help of immobilized mannose 6-phosphate receptors (19). However, this assay is handicapped by the limited availability of the receptor and by the lack of discrimination between ligands containing exclusively the uncovered or a mixture of the uncovered and covered phosphate residues.

The simplest biochemical diagnosis in mucolipidoses II and III is performed by examining an elevation of the activity of lysosomal enzymes in serum (20) or

deficiency of the activity of lysosomal enzymes in cultured fibroblasts or amniotic cells (21). An alternative is the determination of the activity of N-acetylglucosaminyl phosphotransferase, but this is rather laborious and has been performed in a few laboratories only. By measuring the activity of this enzyme with a lysosomal enzyme and with α-methyl mannoside as substrate, a variant of mucolipidosis III may be recognized in which the transferase has normal catalytic activity but a low affinity to lysosomal enzymes (22, 23).

Until now all studied cases of mucolipidoses II and III have been explained by a deficiency of the N-acetylglucosaminyl phosphotransferase. This enzyme is either completely or partially absent or inactive (4, 5, 7, 14, 24), or is unable to recognize lysosomal enzymes (22, 23). The partial deficiencies with 3 to 30% residual activity usually correspond to mucolipidosis III. However, the lower threshold may vary depending on the ethnic origin of the patients and the method used in the enzyme assay. *Okada* et al. have found up to 13% residual transferase activity in a group of Japanese mucolipidosis II patients (24). In their study no correlation between the residual phosphorylating activity and the clinical severity of the disease could be established. It may be expected that the clinical presentation of mucolipidoses II and III mirrors not only the ability of the cells to phosphorylate but also to uncover and target the lysosomal enzymes to lysosomes. In the present work we show that the method of metabolic labelling of cells and immunoisolation of lysosomal enzymes labelled with [^{32}P]phosphate, which has been used previously to discriminate between mucolipidoses II and III (7), can be easily extended to determine the uncovering ratio in both intracellular and secreted lysosomal enzymes.

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